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Comparing the endothelialisation of extracellular matrix bioscaffolds with coated synthetic vascular graft materials.

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1. Introduction

Existing synthetic vascular grafts have unacceptably high failure rates when replacing small diameter infrapopliteal vessels [1]. The lack of a confluent endothelial lining is repeatedly cited as the most common cause of conduit failure [2, 3]. In vitro graft endothelialisation is an emerging method, which has been shown in several long-term human clinical trials to significantly enhance the patency rates of small caliber synthetic grafts [4-7]. In this technique, autologous endothelial cells (ECs) are harvested from superficial veins or adipose tissue and seeded onto the graft lumen prior to implantation. Existing synthetic graft materials are however, poor substrates for cell culture and must be combined with coatings to promote cellular proliferation and adhesion [8, 9]. To date, a plethora of graft coatings have been investigated with no common consensus as to the most efficacious. The aim of this study is to compare the endothelialisation of the most commonly employed graft coating clinically; fibrin-coated expanded polytetrafluoroethylene (ePTFE) with novel decellularised xenogenic extracellular matrix (ECM) scaffolds. ECM scaffolds support the growth of several cell types [10-12] and have already proven successful in reconstructing a wide range of specialised tissues [13-15]. The ECM scaffolds we are examining are derived from the porcine urinary bladder wall - Urinary Bladder Matrix (UBM) and porcine jejunum - Small Intestine Submucosa (SIS). We hypothesise that an intact ECM scaffold would better approximate the natural vascular wall architecture than existing graft coatings and provide a superior substrate for in vitro endothelial seeding. We are specifically examining cellular viability, phenotype, attachment, growth and morphology.

2. Methods

2.1 Preparation of scaffolds

2.1.1 Urinary Bladder Matrix (UBM) Bioscaffolds

A urinary bladder was obtained from market-weight pigs following euthanasia. Urothelial cells were removed by soaking the bladder in normal saline solution. The bladder was then incised via its apex and halved. The external layers of the bladder wall (tunica serosa, tunica muscularis externa, tunica submucosa, and the muscularis mucosa) were removed by mechanical delaminated. The remaining bilayered material including the basement membrane of the tunica mucosa layer (luminal surface) and the subadjacent tunica propria layer (abluminal surface) constitute UBM. Decellularisation was achieved by soakage in 0.1% (v/v) peracetic acid, 4% (v/v) ethanol and 95.9% (v/v) sterile water. The sheet was then soaked in distilled water with phosphate buffered saline to return the pH to neutrality. Finally UBM was terminally disinfected by 10-kGy-gamma irradiation.

2.1.2 Preparation of Small Intestine Submucosa (SIS) Bioscaffolds

SIS was harvested from the porcine jejunum. Sections of rinsed jejunum were longitudinally spit to form an elongated sheet. The superficial mucosal and external muscular layers with surrounding serosa were extracted by physical delamination. The remaining layers; submucosa, muscularis mucosa and basilar layers of the mucosa, the most superficial of which is the stratum campactum (the luminal layer), constitute SIS. The side from which the muscular layers were

removed is the abluminal surface. This tissue was rinsed with phosphate buffered saline (pH = 7.0) and distilled water to lyse any remaining cells and remove residual cellular debris. This was then sterilised with 0.1% peracetic acid and 20% ethanol and finally 1.5 MRad gamma irradiation.

2.1.3 Fibrin Gel preparation

The formation of fibrin gel has already been documented [16]. The production method is summarized here. Fibrinogen in tissue buffered saline (TBS) solution at a concentration of 10mg/ml was prepared. Thrombin solution was then made to get the final concentration of 40IU/ml of thrombin. Prior to use, 75ul of 50mM $CaCl_2$ in tissue buffered saline was added to 75µl of 40IU/ml thrombin and 350µl of TBS. Then 500µl of fibrinogen solution was added and mixed with gentle shaking in 24 well plates. The gel was left for one hour to polymerise in an incubator at 37°C with 5% CO_2 and 95% O_2 environment. Fibrin glue was applied evenly to the graft surface using a sterile syringe.

2.2 Sample preparation

2.5cm diameter circular segments of each material was placed under sterile conditions between two stainless steel rings with inner diameters of 2.2cm such that 3.8 cm^2 of the luminal graft surface was exposed. The luminal surfaces of UBM and SIS were used for seeding of cells.

2.3 Cell culturing technique

Human umbilical vein endothelial (HUVEC) cell lines were purchased from Cascade Biologics/Invitrogen ®. HUVEC culture medium 200 was used and supplemented with low serum growth supplement (Cascade Biologics/Invitrogen). The media was replaced every 48 hours untill cells reached confluency. The cells were then split in a ratio 1:3 with 3mls buffered saline solution containing 0.25% trypsin and 0.09% ethylenediaminetetraacetic acid (EDTA).

2.4 Seeding Protocols

2.5cm diameter discs of each material were placed in six well culture dishes. The Sterile stainless-steel culture rings were placed over the scaffolds to prevent cell leakage. The cells were seeded at a density of 7×10^4 cells per cm^2 in 3mls of culture media. The constructs were then left in an incubator for two hours to allow for cell adhesion. Thereafter the chambers were flooded with media and the constructs were returned to the incubator.

2.5 Cellular Viability

The cytotoxicity of the constructs to HUVECs was assessed by determining cellular viability using a live/dead viability assay (Invitrogen™). The methodology of this assay has been prescribed previously [17]. Images were captured using a Nikon Eclipse TE200 inverted microscope.

2.6 Immunofluorescence analysis of von Willebrand Factor (VWF)

Endothelial cells seeded on each substrate were fixed with 3.7% para formaldehyde in phosphate buffered saline (PBS). These were then rinsed with PBS and mixed for 20 min with 0.27% NH4Cl/0.38% glycine in PBS and permeabilised with Triton X- 100 (0.5%) in PBS. Von Willebrand Factor was confirmed via fluorescein-labeled Antibodies-conjugated mouse anti-human VWF antibodies (2 μgmL^{-1}).

2.7 Cellular Attachment

Cellular adhesion was determined by examining the percentage of attached cells over time, seventh passage cells were used in each experiment. Uniform passages HUVECs (P7) of 7×10^4 cells per cm^2 were seeded separately onto each material surface in a drop wise manner and incubated for a maximum of 120 minutes. At 30 minute intervals, the surface of the constructs were rinsed with 5ml phosphate buffer solution (PBS) containing 0.4% v/v trypan blue to wash off any unattached cells. The detached cells were counted with a hemocytometer and expressed as a fraction of the original seeding density.

2.8 Cellular Proliferation

Cellular proliferation was assessed on days 1,3,5 and 7 using the tetrazolium compound 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-

sulfophenyl)-2H-tetrazolium (MTS) colorimetric assay. This assay has previously been described [18].

2.9 Scanning Electron Microscopy (SEM)

Each construct with HUVECs was primarily fixed with 2.5% glutaraldehyde/ paraformaldehyde phosphate buffer solution. After primary fixation, the constructs were post-fixed in 2% Osmium Tetroxide then dehydrated through a graded series of acetones up to 100%. After critical point drying, the specimens were mounted onto aluminium stubs and then coated with gold. Samples were then analyzed using the Scanning Electron Microscope - JEOL 5510.

2.10 Statistical Analysis

Statistical analyses were conducted using SPSS 22.0. Data was summarised using means and standard deviations. Statistical significance was determined as $p \leq 0.05$. Each quantitative experiment was performed in triplicate. For cell attachment, a 5x3 factorial analysis of variance (ANOVA) was conducted to examine the effects of materials, time and the interaction between these two factors. For cell proliferation, a 5x5 factorial analysis of variance was conducted to examine the effects of materials, test days and the interaction between these two factors. Where a significant interaction was found, this was explored using simple main effects analysis. Simple main effects of materials at each time (or test day) were conducted. The significant simple main effects of materials were further analysed by pairwise comparisons employing a Bonferroni adjustment for multiple comparisons. For cell attachment, for

each material the simple main effects of time were explored. Significant simple main effects of time were further analysed by pairwise comparisons using a Bonferroni adjustment for multiple comparisons. The underlying assumption of Normality was examined using Normal probability plots and the Shapiro-Wilk test. The homogeneity of variance test was examined by plotting the residuals of the model against the fitted values and the Levene's test.

3. Results

3.1 Cellular Viability

Live/Dead assays showed that synthetic grafts, fibrin/ePTFE hybrid and the ECM scaffolds all support the viability of HUVEC cells in static culture, demonstrating that neither the scaffolds nor their coatings were cytotoxic to the HUVECs.

3.2 Validation of endothelial cells

In the immunofluorescence staining, intracellular VWF of HUVECs was confirmed on all materials. Figure one shows ECs cultured on Dacron expressing VWF, confirming that ECs were functioning appropriately. Cells cultured on the other scaffolds were also shown to be expressing VWF.

3.3 Cellular Attachment

Data showing the proportion of cells attaching to each substrate over 120 minutes in culture is tabulated in figure two. For cell attachment, the 5x3 ANOVA indicated significant main effects of materials ($p<0.001$) and time ($p<0.001$), and a significant interaction between materials and time ($p=0.016$). The material by time interaction effect was analysed using simple main effects analysis. For each time point, significant differences between materials were found ($p<0.001$, for all). These significant simple main effects of materials were further analysed by pairwise comparisons using the Bonferroni adjustment for multiple comparisons. ECs seeded on both ECM scaffolds and fibrin-modified ePTFE achieved statistically higher attachment efficiency at each time point when compared to both synthetic graft materials ($p \leq 0.001$). The adhesion rates between the ECM scaffolds and the fibrin-coated ePTFE was statistically similar at each time point ($p = 1.00$). For each material, significant differences between time points were found ($p<0.001$, for all). These significant simple main effects of time were further analysed by pairwise comparisons using the Bonferroni adjustment for multiple comparisons. For all materials examined, cell attachment was significantly higher at 90 ($p = 0.001$) and 120 minutes ($p = 0.001$) compared to 60 minutes. There was no difference in attachment rates beyond 90 minutes ($p > 0.05$).

3.4 Cellular Proliferation

Cell Proliferation for each material is illustrated in figure three. For cell proliferation, the 5x5 ANOVA indicated significant main effects of materials ($p < 0.001$) and test days ($p < 0.001$), and a significant interaction between materials and test days ($p < 0.001$). The material by test day interaction effect was analysed using simple main effects analysis. For each test day, significant differences between materials were found ($p < 0.001$, for all). These significant simple main effects of materials were further analysed by pairwise comparisons using the Bonferroni adjustment for multiple comparisons. For days 3,5,7 and 9 cell proliferation was significantly higher on UBM, SIS and fibrin/ePTFE when compared to Dacron and ePTFE ($p \leq 0.001$). From day three on, cells grew faster on the ECM scaffolds when compared to fibrin-coated ePTFE ($p \leq 0.001$). ECs grown on ECM scaffolds achieved confluency the fastest (day seven) with no significant growth after this time ($p = 1.000$). There was no difference in cell proliferation at any time point between UBM and SIS ($p = 1.000$). There was also no difference between ePTFE and Dacron at any time point ($p = 1.000$). Cells grown on fibrin/ePTFE hybrid or uncoated synthetic grafts failed to reach confluency by the end of the study (day nine).

3.5 Cellular Morphology

On uncoated ePTFE and Dacron cells appeared as small spheroid shapes with single point contact to the material (Fig. 4a). They were inhomogeneously distributed on the surface and aggregated in clusters. As illustrated, cell seeded onto ePTFE did not cover the prosthetic fibers uniformly and produced isolated clumps of cells with large sections of fibers exposed. Findings on Dacron were similar. Fibrin/ePTFE hybrid

appeared to be almost completely covered by a homogeneous layer of cells. The cells were well spread on the surface with a large mean cell–material contact area. Cells grown on ECM scaffolds produced a similar pattern, a homogeneously distributed and well-spread monolayer, demonstrating a cobblestone morphology (Fig. 4b).

4. Discussion

The principle requirements for in vitro endothelialisation are rapid cell proliferation and strong cellular attachment to minimise culture times and resist the haemodynamic forces upon implantation. We found cell attachment to Dacron was negligible, possibly due to the high porosity of the material resulting in leakage of the seeding solution (Fig. 2). Uncoated ePTFE displayed similarly poor attachment rates despite its smoother surface and smaller pore size. All biological scaffolds including fibrin/ePTFE hybrid supported stronger adhesion when compared to uncoated ePTFE and Dacron at each time point ($p \leq 0.001$). The finding that fibrin enhances endothelial attachment are constant with the results of Zilla et al. [19]. Fibrin has excellent biocompatibility properties with a high affinity for biological surfaces [20]. It supports angiogenesis during wound healing and possesses several cellular attachment sites [21].

Both ECM materials displayed equally high adhesion rates despite the different composition and topography of SIS and UBM. SIS possesses an interstitial like surface, while UBM contains a fully intact basement membrane [22]. While investigating graft endothelialisation, Baker et al. reported that endothelial cells attached equally well to the basement surface (collagen types IV and V) and interstitial surface (collagen types I and III) of human amnion [23]. The exact

attachment mechanisms of endothelial cells to ECM remains to be elucidated but is likely to be multifactorial and complex. Adhesion proteins contained in ECM scaffolds, such as fibronectin, collagen type I and laminin are widely recognised as important attachment substrates for endothelial cells, encouraging growth and "sprout" formation [24, 25]. Growth factors identified in SIS and UBM enhance and may play a synergic role in promoting cellular attachment. Fibroblast growth factor 2 (FGF-2), transforming growth factor beta (TGF-β) and vascular endothelial growth factor (VEGF) found in ECM are proven to encourage the adherence of endothelial cells [26, 27]. These growth factors are bound to ECM proteoglycans and glycosaminoglycans, which protect them from degradation and enhance their presentation to occupying cells [28]. Importantly these growth factors have been shown to survive the production and sterilisation steps in preparing ECM scaffolds for medical use [29,30]. In the past, researchers have struggled to integrate purified forms of growth factors into synthetic scaffolds [31]. Difficulties in the release and formulation of the correct combinations have lead to disappointing clinical results. In natural ECM biomaterials, these growth factors appear to be present in the correct concentrations, isoforms and three-dimensional ultra structures [28]. As well as the components of ECM scaffolds, the topography may also influence cellular adhesion. Several studies have reported changes in cellular behaviour depending on the roughness of their substrates. This has lead to nanoscale engineering to influence cellular orientation, growth, attachment and migration [32, 33]. Miller et al. reported the improved attachment and growth of ECs on poly (lactic-co-glycolic acid) (PLGA) materials by mimicking the surface roughness of natural extra-cellular matrix [34]. The luminal surface of SIS and UBM comprise a dense compact basement membrane like surface following the removal of the epithelial layer. The topography found in

ECM, may provide cells with a more natural nanosurface, resembling that found in vivo and positively influencing cell growth and attachment.

The morphological difference between cells grown on synthetic and biological grafts (SIS, UBM, fibrin/ePTFE) is striking. On ePTFE and Dacron (Fig. 5a), cells appear as small spheroid shapes with single point contact to the material. This morphology suggests that these cells have not yet acclimatized to the substrate and are poorly attached, essentially in a quiescent phase. They are inhomogeneously distributed on the surface and aggregate in clusters leaving large sections of graft fibers exposed. Cells grown on the biological substrates including the fibrin/ePTFE hybrid exhibited larger, flatter cell shapes with increased cytoplasmic extensions to the materials. These cells have greater numbers of attachment bonds to the substrate, thus increasing attachment strength. All cells examined on the biological substrates were beginning to form the typical cobblestone monolayer morphology as seen in vivo which is known to correlate with high levels of attachment and facilitate enhanced cellular proliferation [35]. Flattened phase endothelial cells are also in a shape most capable of allowing the streamlining of blood flow thus minimizing cell loss at implantation.

In vitro endothelialisation not only requires adequate cellular attachment, but also rapid proliferation. Slow proliferation increases incubation times leading to higher rates of contamination and production costs. This delays implantation and increases the initial cell harvest density required. Our results demonstrated that all materials studied, supported cellular proliferation to some extent (Fig. 3). Although cells grown on synthetic grafts remained viable and displayed consistent yet slow growth, they did not achieve confluency by the end of the study (day nine). The high porosity and poor cellular attachment may have initially delayed

cell-cell contact formation and therefore had a negative impact on cell growth. Furthermore, the fibrillar interstices of ePTFE and the Dacron fibers may have hindered cellular migration. Despite the excellent attachment rates achieved with fibrin-coated ePTFE, this success was not replicated when examining proliferation. The reason for this is not fully understood. Theoretically, fibrin is an excellent scaffold, supporting angiogenesis and tissue repair in vivo [36]. It also releases fibrinopeptides, which are known to be mitogenic for ECs [21]. Henrich et al. examined two types of fibrin glue and also discovered similarly high levels of attachment but weak proliferation [37]. The delayed proliferation of cells on fibrin/ePTFE could be explained by the dense matrix formed by the polymerisation of fibrinogen. Several studies have cited that this dense matrix could inhibit the migration and proliferation of cells [38,39]. Although fibrin did increase the proliferation rate when compared to uncoated ePTFE, this was insufficient to form a confluent endothelium within the time frame of this study.

Of all the vascular materials studied, cells proliferated fastest on ECM scaffolds with both reaching confluency at day seven. Each had statistically higher cell numbers from day three onward compared to all other scaffolds. It is generally accepted that cellular growth on biomaterials is highly reliant on the topography and composition of the substratum [40]. In vivo, ECM is crucial during angiogenesis, which involves endothelial growth and spreading [41-43]. ECM is rich in substances known to enhance angiogenesis such as the growth factor VEGF [27] and functional proteins such as laminin and fibronectin, which accelerate endothelial proliferation. Glycosaminoglycans present in ECM bind to growth factors and cytokines and encourage cellular growth. In vivo, the luminal surface of SIS and UBM support a continually dividing cellular layer; the small intestine enteriocytes in

SIS and transitional epithelium in UBM. These layers are repeatedly subjected to mechanical and infectious trauma, hence it is reasonable to assert, that ECM from such areas has evolved to maximise cellular growth and differentiation.

ECM materials are at the forefront of biomaterial research and have been successfully used in a diverse range of medical applications [13-15]. As our understanding of these materials increases, it is becoming more apparent that they possess many properties that would make them ideal for use in vascular tissue engineering. Extensive research has been carried out on ECM materials; particularly SIS and the results suggest that these scaffolds fulfill many of the design criteria proposed for a novel vascular graft. In terms of mechanical characteristics, SIS has an axial strength greater than that of the canine carotid artery [44]. It has enhanced compliance, which more closely approximates that of an artery when compared to ePTFE or Dacron grafts [44]. The compliance of ECM grafts could reduce intimal hyperplasia and this has been born out in several studies [45,46]. Burst pressure studies on SIS show it to be easily capable of resisting the continuous haemodynamic stresses in vivo [44] and animal studies of up to five years have not shown aneurysmal formation or graft failure [47].

6. Conclusion

The composition of intact ECM appears to be an ideal substrate for promoting rapid endothelialisation by combining firm cellular anchorage and rapid cell expansion. In a clinical setting where a finite number of cells are required to form a complete monolayer within a short time frame, firm cellular attachment combined with rapid endothelialisation will be important criteria in determining the success of this

emerging technology. Future studies would involve alternate cell lines including endothelial progenitor cells or microvascular endothelial cells and examining their behaviour when exposed to sheer stresses. In conclusion this work suggests that ECM materials are promising scaffolds for small vessel tissue engineering.

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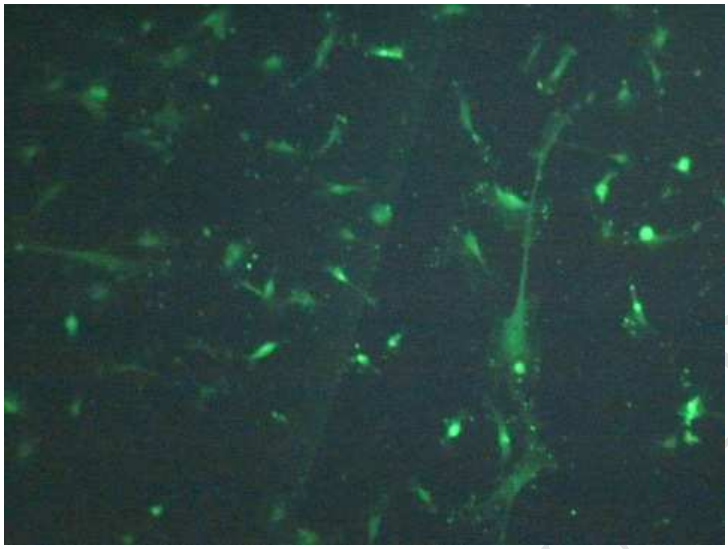

Fig. 1. Endothelial Cells expressing VWF on Dacron material. Cells grown on the other scaffolds were also shown to express VWF. Original magnification × 20 microscope objective.

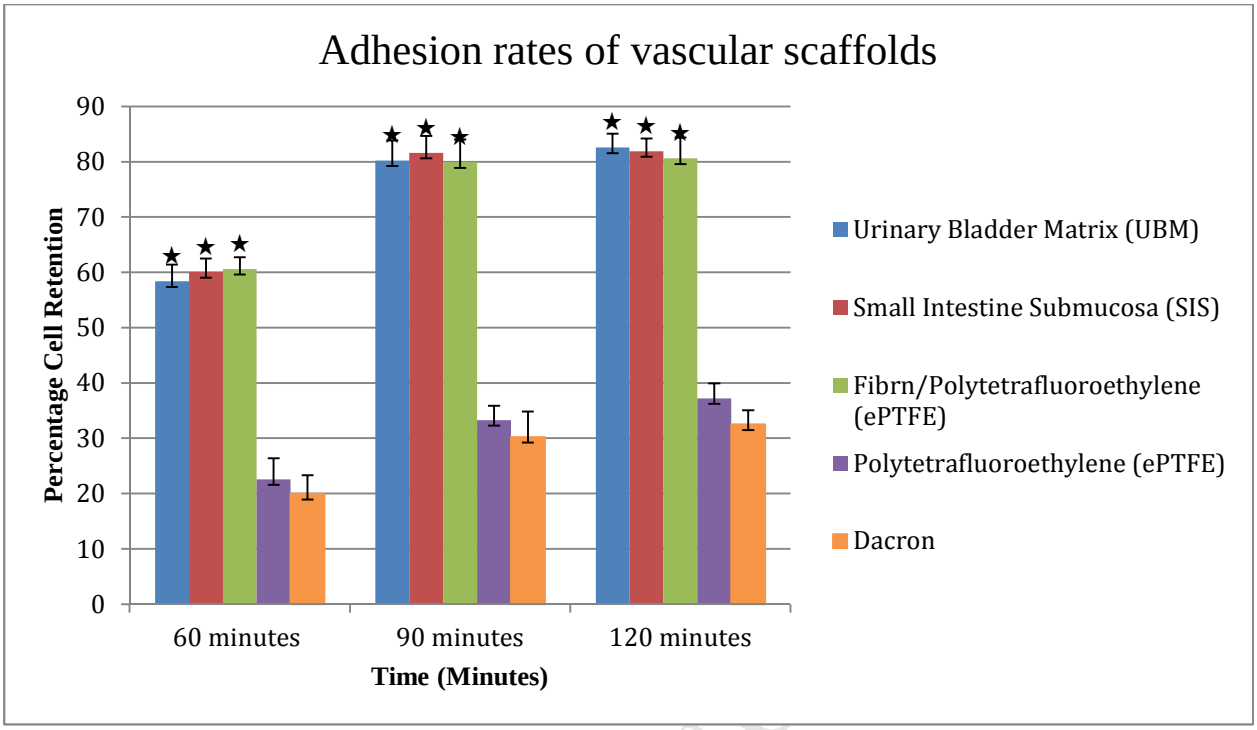

Fig. 2. Comparison of the percentage of HUVEC attached on different substrates. The values are mean of three replicates in the case of each matrix (+/- 1 Standard Deviation) ★ denotes statistical significance ($p \leq 0.001$) of attachment rates on UBM, SIS and Fibrin/ePTFE grafts when compared to uncoated synthetic graft materials; ePTFE and Dacron.

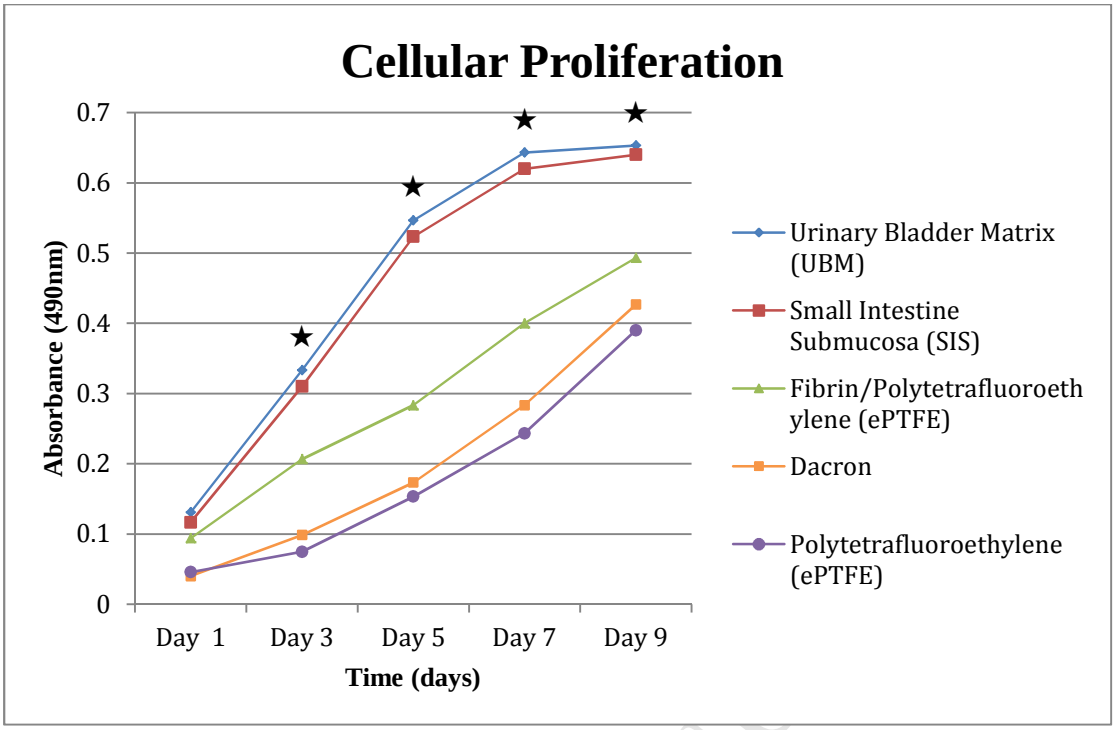

Fig. 3 Cellular Proliferation Graph. The values are mean of three replicates in the case of each scaffold. ★ denotes statistical significance ($p \leq 0.001$) of each result on ECM scaffolds relative to fibrin/ePTFE hybrid graft materials.

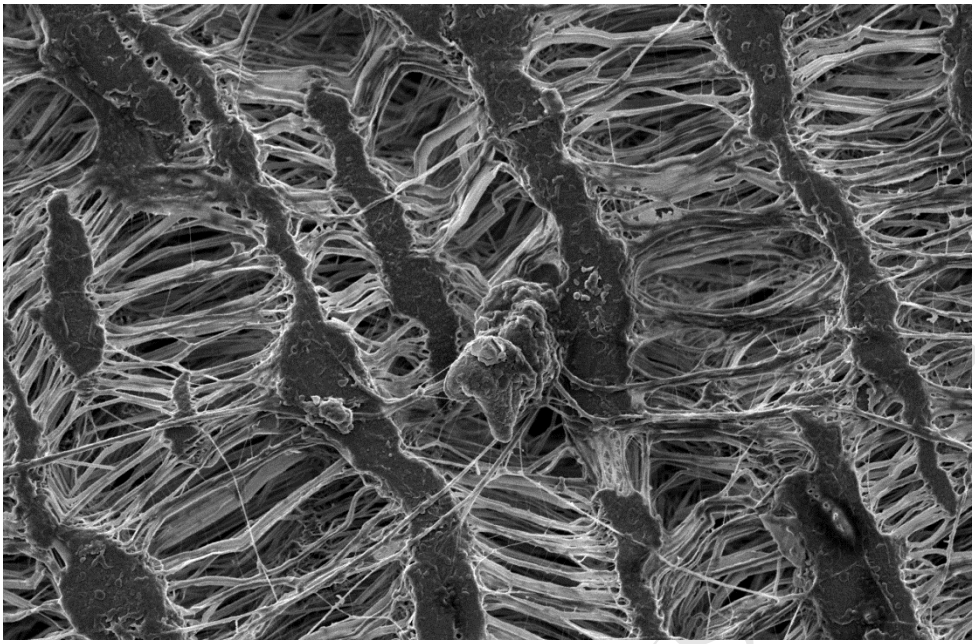

Fig. 4a. Endothelial Cells on ePTFE SEM × 1300 Magnification. Findings with Dacron were similar.

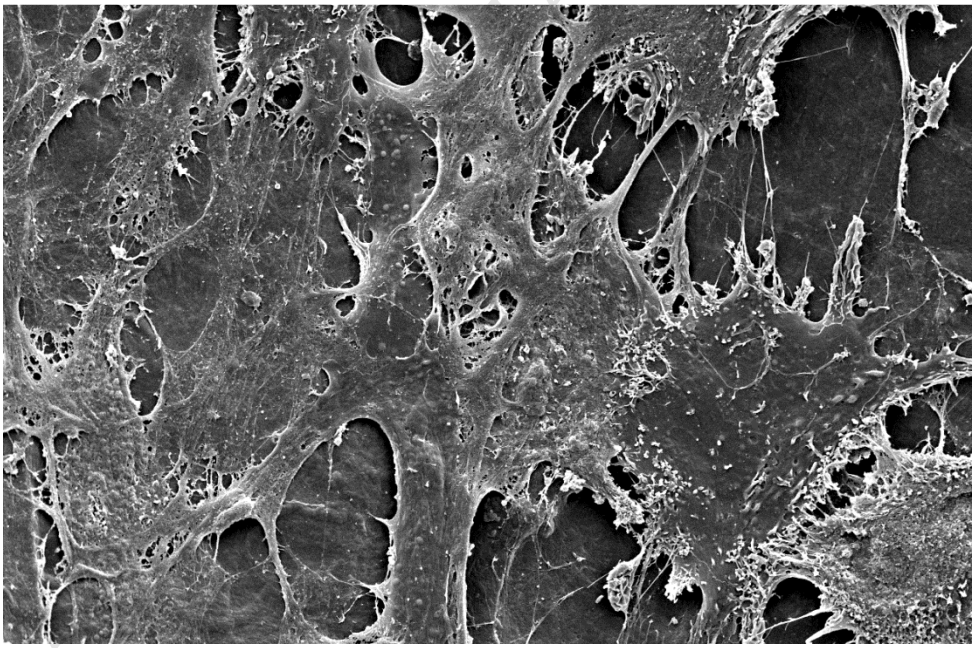

Fig 4b. Endothelial Cells on SIS. SEM × 1300. Magnification. Findings on UBM and fibrin/ePTFE were similar.